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The V Protein of Human Parainfluenza Virus 2 Antagonizes Type I Interferon Responses by Destabilizing Signal Transducer and Activator of Transcription 2

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Type I interferon (IFN) induces antiviral responses through the activation of the ISGF3 transcription factor complex that contains the subunit proteins STAT1, STAT2, and p48/ISGF3γ/IRF9. The ability of some human paramyxoviruses to overcome IFN actions by specific proteolysis of STAT proteins has been examined. Infection of cells with type 2, but not type 1 or type 3 human parainfluenza virus (HPIV) leads to a loss of cellular STAT2 protein. Expression of a single HPIV2 protein derived from the V open reading frame blocks IFN-dependent transcriptional responses in the absence of other viral proteins. The loss of IFN response is due to V-protein-induced proteolytic degradation of STAT2. Expression of HPIV2 V causes the normally stable STAT2 protein to be rapidly degraded, and this proteolytic activity can be partially alleviated by proteasome inhibition. No V-protein-specific effects on STAT2 mRNA levels were observed. The results indicate that the V protein of HPIV2 is sufficient to recognize and target a specific cellular transcription factor for destruction by cellular machinery.

Key Words: STAT; ISGF3; interferon; paramyxovirus; V protein; HPIV2; proteolysis.

INTRODUCTION

Cellular response to virus infection involves both innate and adaptive host immune functions. Interferons (IFNs) have long been recognized as the mediators of innate antiviral responses. Type I IFNs, IFNα and IFNβ, are the principal antiviral cytokines produced by mammalian cells and function directly on target cells by blocking viral replication (Isaacs and Lindemann, 1957; reviewed in Goodbourn *et al.,* 2000).

IFN-dependent interference with viral infection relies on a failure of viral mRNA translation or of viral nucleic acid replication, but the molecular basis for the antiviral state is still not completely understood. It is known that the establishment of the cellular antiviral state requires mRNA and protein synthesis following IFNα treatment. IFNα/β-induced proteins that contribute to the antiviral state are known and these proteins are often the target of virus IFN antagonism (reviewed in Goodbourn *et al.,* 2000). Well-studied examples include activation of such proteins as the double-stranded RNA-dependent protein kinase (protein kinase R), the double-stranded RNA-dependent 2′–5′ oligoadenylate synthetase–RNAse L system, and the IFN-induced Mx proteins. The effects of IFNα/β in causing the overall antiviral state depend on the synthesis of one or more of these proteins and additional other pathways that have been uncovered in studies of mice deficient in all three of these antiviral effectors (Zhou *et al.,* 1999).

Analysis of the promoters of many IFNα/β-stimulated genes revealed a DNA response element specific for Type I IFN induction, the IFN stimulated response element (ISRE; reviewed in Darnell, 1997; Horvath, 2000). ISGF3 was identified as the protein factor that binds to this response element and was found to contain three proteins from two transcription factor families. Two subunits are members of the signal transducer and activator of transcription (STAT) family. The IFNα/β-activated factor (ISGF3) contains a heterodimer of STAT1 and STAT2 complexed with a third protein, a member of the interferon regulatory factor family referred to as ISGF3γ, p48, or IRF9. The IRF9 protein is an essential component of ISGF3 that confers DNA binding specificity to the ISGF3 complex (Levy *et al.,* 1989) and provides specific protein binding recruitment sites for the STAT1 and STAT2 proteins (Horvath *et al.,* 1996; Lau *et al.,* 2000; Martinez-Moczygemba *et al.,* 1997).

STAT1 and STAT2 are required for ISGF3 signaling, but are not redundant in this pathway. STAT1 protein is a

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component of both the Type I IFN-induced ISGF3 complex and the Type II IFN-induced GAF complex and consequently cells lacking STAT1 have no antiviral response to either IFN (Horvath and Darnell, 1996). Full IFNα/β antiviral activity requires only that STAT1 be phosphorylated on tyrosine, but either STAT1α or STAT1β (a form of STAT1α lacking the 38-amino-acid transcriptional activation domain) will suffice (Horvath and Darnell, 1996). These results indicate that while STAT1 is indispensable for ISGF3-induced antiviral effects, STAT2 plays a crucial role as the transcription activating protein component in ISGF3. STAT2 is clearly essential for IFN responses and ISGF3 function, as deficiencies in STAT2 can disrupt ISGF3 signaling and lead to decreased expression of antiviral genes (Leung *et al.,* 1995; Qureshi *et al.,* 1996).

As the IFNα/β system represents an early and crucial step in anti-viral immunity, it is not surprising that many viruses have evolved strategies to impede the actions of this cytokine (reviewed in Goodbourn *et al.,* 2000). The ability of a virus to antagonize the IFNα/β pathway can have dramatic consequences for the success of infection, as illustrated in several cases where the IFN antagonizing genes were deleted to form attenuated viruses (for example, Bergmann *et al.,* 2000; Kitajewski *et al.,* 1986). The virulence of a specific strain can be correlated with its susceptibility to the antiviral effects of IFNα/β, and evolution of strategies to enhance IFNα/β resistance are expected to lead to highly infectious viruses and/or persistent infections. A wide variety of viruses fight the IFNα/β system at a variety of steps, in many cases at an early point upstream of gene activation, antagonizing both IFN responses and IFN production (Bergmann *et al.,* 2000; Kitajewski *et al.,* 1986; Talon *et al.,* 2000).

Negative-strand RNA viruses of the family *Paramyxoviridae* have evolved specific proteins that directly suppress IFN signaling by lowering the concentration of cellular STAT proteins. For example, Sendai virus has been demonstrated recently to block IFN responses through the actions of its C proteins (Garcin *et al.,* 1999). Expression of this single viral protein is sufficient to cause a loss of STAT protein inducibility at the transcriptional level. The mechanism of STAT1 transcriptional suppression is unknown, but is suspected to involve interaction of C proteins with cellular factors (Garcin *et al.,* 1999, 2000). A second paramyxovirus, SV5, was demonstrated recently to evade IFN antiviral responses by specifically targeting the transcription factor STAT1 for proteolytic degradation (Didcock *et al.,* 1999). This destruction of STAT1 was found to be mediated by the sole expression of a single virus-encoded protein derived from the V open reading frame (Didcock *et al.,* 1999). Subsequent analysis indicated that while most paramyxoviruses can block IFN signaling, the ability to degrade STAT1 was a unique property of SV5 (Young *et al.,* 2000). Somewhat surprisingly, it was also found that in cells infected with human parainfluenza virus 2 (HPIV2), STAT1 was not degraded, but rather a loss of STAT2 was observed (Young *et al.,* 2000). The mechanism of STAT2 degradation or the viral gene responsible for catalyzing its destruction was not investigated in this study.

As a first step in understanding the specificity of STAT protein degradation by paramyxoviruses and defining the cellular machinery involved in this process, we investigated the ability of the HPIV2 V protein to mediate loss of STAT2 protein in the absence of other viral proteins. Our results corroborate the finding that infection of cultured cells with HPIV2, but not with HPIV1 or HPIV3, causes a dramatic reduction of STAT2 protein steady-state levels. Moreover, we demonstrate that the expression of HPIV2 V protein from a cDNA clone is sufficient to abolish IFN responsive transcriptional activity. Our data illustrate that expression of the HPIV2 V protein in cultured cells did not influence STAT2 mRNA abundance, but instead was found to reduce steady-state STAT2 protein levels by increasing its degradation rate, leading to uncharacteristic STAT2 instability. Interestingly, this proteolysis is only partially alleviated by proteasome inhibitors, suggesting that alternatives to the ubiquitin–proteasome degradation system may be responsible for the V protein actions.

RESULTS

Effects of HPIV infection on steady state STAT1 and STAT2 protein levels

To examine the effects of human parainfluenza viruses on steady-state levels of STAT proteins, cultured cells were infected with human parainfluenza viruses (HPIV) 1–3. At 9 h postinfection, cells were lysed in whole cell extract buffer and subjected to SDS–PAGE and immunoblotting with antisera for STAT1 and STAT2. Despite a small reduction in STAT1 in HPIV1-infected cells, only minor differences in STAT1 levels were observed. STAT2 was found to be stable during infection with HPIV1 and HPIV3. In fact, a stronger STAT2 signal was detected following infection with HPIV1 and HPIV3 compared to uninfected cells, possibly indicating induction of STAT2 expression by these viruses. In contrast, HPIV2 infection caused a nearly complete loss of STAT2 protein (Fig. 1). This result indicates that HPIV2 infection induces a reduction in the STAT2 expression level in agreement with the prior data (Young *et al.,* 2000).

Expression of HPIV2 V protein from transfected cDNA

The degradation of STAT1 as a result of SV5 infection was found to be mediated by the V protein. The HPIV2 virus codes for a V protein that is 44% identical in amino acid sequence to the SV5 V protein. This high degree of

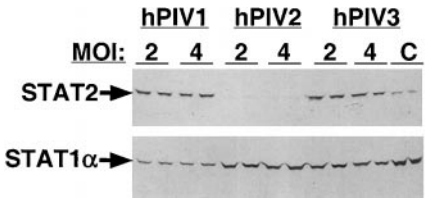

FIG. 1. STAT2 protein level decreases during HPIV2 infection. CV1 cells were infected with human parainfluenza viruses 1–3 at the indicated multiplicity of infection (m.o.i.) for 9 h. C, control uninfected cells. Whole cell lysates (50 μg protein) were separated by SDS–PAGE and transferred to nitrocellulose filters for immunoblotting with antisera specific for STAT1 or STAT2.

identity is spread throughout the length of the protein with especially high conservation in the C-terminal cysteine-rich domain. The extensive homology between the two proteins suggested that the HPIV2 V protein might be involved in the observed loss of STAT2. As a first step toward understanding the molecular events involved in virus-induced reduction of STAT protein stability, the role of HPIV2 V in catalyzing the loss of STAT2 was investigated.

For mammalian cell expression, the V protein cDNA was subcloned into the mammalian expression vector, pRcCMV, under control of a constitutively active cytomegalovirus promoter. To verify the integrity of our construct, expression of the V protein was tested in human embryonic kidney 293 T cells. Duplicate plates of cells were transfected with control vector or HPIV2 expression vector and extracts were prepared 48 h later. The extracts were separated by SDS–PAGE and transferred to nitrocellulose filters. Probing these blots with an antiserum generated against HPIV2-infected cells resulted in recognition of a novel band in the V-transfected cells migrating at the position of approximately 27–30 kDa on the SDS gel, in general agreement with the theoretical molecular weight of the HPIV2 V protein calculated from the amino acid sequence (24,150 kDa; Fig. 2A) (Ohgimoto *et al.,* 1990).

Expression of HPIV2 V protein leads to loss of cellular STAT2 protein

To determine the effect of HPIV2 V protein expression on steady-state levels of STAT2 protein, cells were transfected with pRcCMV vector or pRcCMV HPIV2 V and extracts prepared at 48 h posttransfection following a 6-h treatment with or without IFNα. Samples were separated by SDS–PAGE, transferred to nitrocellulose, and probed with a STAT2-specific antiserum. STAT2 levels were consistently reduced to ~30–50% upon HPIV2 V protein expression compared with controls and IFN treatment did not alter this effect of V protein expression (Fig. 2B). A similar reduction of STAT2 protein levels was observed in all human cell lines tested (including Hela, 2fTGH, and U3A; data not shown), and effects were most apparent in the efficiently transfected 293T cell line shown, where cotransfected GFP expression plasmid was found to be expressed in nearly all cells prior to lysate preparation. Stripping and reprobing with a STAT1-specific antiserum demonstrates no loss of STAT1 protein, indicating that the effect of HPIV2 V is specific for STAT2. This result indicates that the V protein of HPIV2 does alter the accumulation of STAT2 protein, but in the transfection system the loss of STAT2 was not as complete as that observed upon HPIV2 virus infection. The difference between the two systems might reflect fundamental biological differences inherent in expression of the V protein from a transfected CMV promoter-driven expression vector versus that from a native HPIV2 promoter in the context of virus infection. One explanation to account for this effect is that in the transfected cells, newly synthesized STAT2 might replenish the steady-state pool of protein.

Expression of HPIV2 V protein causes a loss of IFN responsive ISGF3 activity

To determine the effect of V protein expression and subsequent loss of STAT2 protein on type I IFN signaling, a luciferase reporter gene assay was used to measure ISRE-dependent transcriptional activity. Cells were transfected with a reporter plasmid containing five copies of the ISG-54 ISRE element upstream of a luciferase reporter gene, a LacZ control plasmid, and either pRcCMV vector or pRcCMV HPIV2 V. Cells were treated with IFN for 6 h prior to lysis and luciferase assay. As a control, in place of the HPIV2 V plasmid, an SV5 V expression plasmid was expressed in alternate plates. The reporter gene has a low basal activity in the absence of IFN

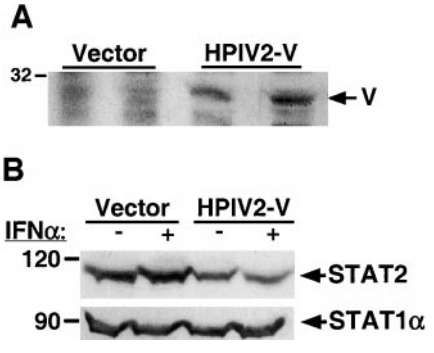

FIG. 2. Expression of HPIV2 V ORF reduces the abundance of cellular STAT2 protein. A. Expression of HPIV2 V protein from cDNA. Duplicate plates of 293T cells were transfected with pRcCMV vector or pRcCMV-HPIV2 as indicated, and cell extracts were prepared 48 h later and subjected to immunoblotting with total HPIV2 antiserum. The positions of the V protein and a 32-kDa prestained molecular weight marker are indicated. B. Expression of HPIV2 V protein reduces the steady-state level of STAT2. Transfected cells were treated with or without IFN-α at 42 h posttransfection, and whole cells extracts were prepared 6 h later. Steady-state STAT1 and STAT2 levels were examined by immunoblotting with specific antiserum.

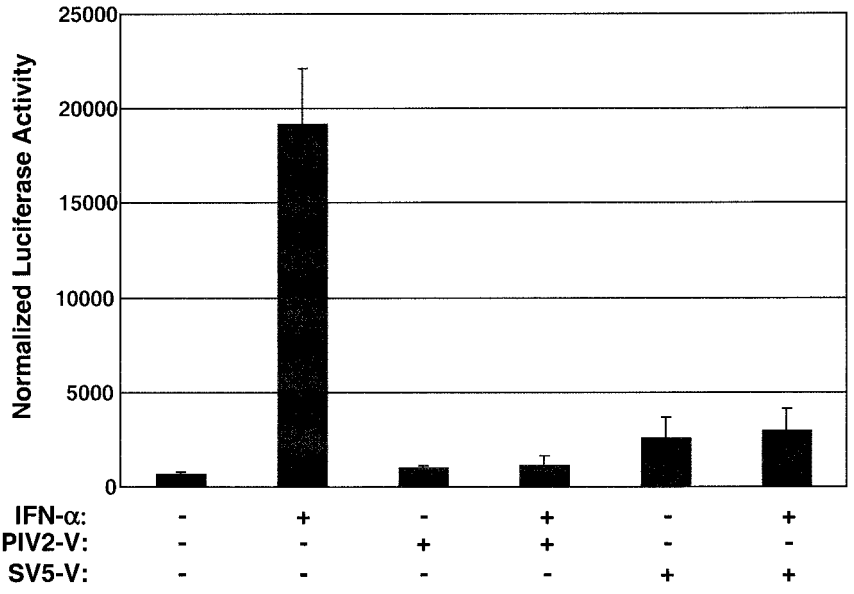

FIG. 3. Inhibition of ISGF3-dependent transcription by expression of HPIV2 V protein. 293T cells were transfected with ISRE-luciferase reporter gene and either empty vector or paramyxovirus V protein expression vectors as indicated. Following IFN stimulation for 6 h, cells were lysed at 48 hpt and subjected to luciferase assays. Results shown are normalized to cotransfected LacZ and represent average values from a triplicate experiment, with standard deviation indicated.

stimulation that is induced approximately 20- to 25-fold after a 6-h treatment with IFNα (Fig. 3). When either HPIV2 V protein or SV5 V protein was cotransfected with the ISRE reporter gene, no IFN inducible activity was observed. These results indicate that both of the paramyxovirus V proteins can antagonize ISRE-dependent transcriptional activation by targeting different components of the ISGF3 complex.

Effect of V protein on STAT2 mRNA levels

To determine the mechanisms responsible for the V-protein-dependent loss of STAT2 protein accumulation and ISGF3 activity, STAT2 mRNA abundance was examined as a possible point of interference by HPIV2 V (Fig. 4). To examine the STAT2 mRNA accumulation level, cells were transfected with pRcCMV vector or pRcCMV HPIV2 V. Immediately following transfection (time 0) and at 12 h posttransfection (hpt), plates were harvested for RNA extraction, and at 12 hpt, the growth medium on remaining plates was replaced. Subsequent samples were harvested at 24 and 36 hpt. Total RNA was extracted and subjected to reverse-transcription and PCR (RT-PCR) with STAT2-specific primers or control primers specific for the cellular enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of radiolabeled deoxynucleoside triphosphate. No products were detected in control reactions carried out in the absence of reverse transcription (not shown). Only minor variations in the level of STAT2 mRNA were observed between individual plates of transfected cells. No significant differences in either STAT2 or GAPDH mRNA expression were observed specifically in response to HPIV2 V protein expression. The level of cotransfected GFP mRNA was quantitated similarly and the data indicate that similar transfection efficiencies were obtained between individual transfected plates in a single experiment (Fig. 4B). This result suggests that the loss of STAT2 protein cannot be attributed to HPIV2 V protein effects on STAT2 mRNA steady-state levels.

Effect of V protein on STAT2 is posttranslational

To measure the rate of STAT2 degradation in the presence and absence of V protein, and to test directly the contributions of newly synthesized STAT2 to the steady-state pool, a time course experiment was carried out. Because endogenous STAT2 protein is very stable with a long half life (Lee *et al.,* 1997), a time course protocol involving cycloheximide (CHX) treatment was used in lieu of inefficient metabolic labeling, based on protocols used for examination of p53 degradation by human papillomavirus proteins (Talis *et al.,* 1998). Cells were transfected with pRcCMV vector or pRcCMV HPIV2 V, medium was changed after 16 h, and CHX was added to half of the plates at 24 hpt. Duplicate plates were harvested at 48, 72, and 96 hpt and cell extracts were subjected to STAT2-specific immunoblotting. An example of representative data from several experiments is shown in Figs. 5A and 5B. The STAT2 protein level was constant in the absence of V protein expression, but CHX treatment resulted in a small decrease in steady-state levels (~20–30% lost by 48 hpt), indicating a low level of STAT2 recycling in the cells. Expression of HPIV2 V protein caused a dramatic reduction of STAT2 after 24 h (~80% loss), after which the STAT2 level remained low but fairly constant, approxi-

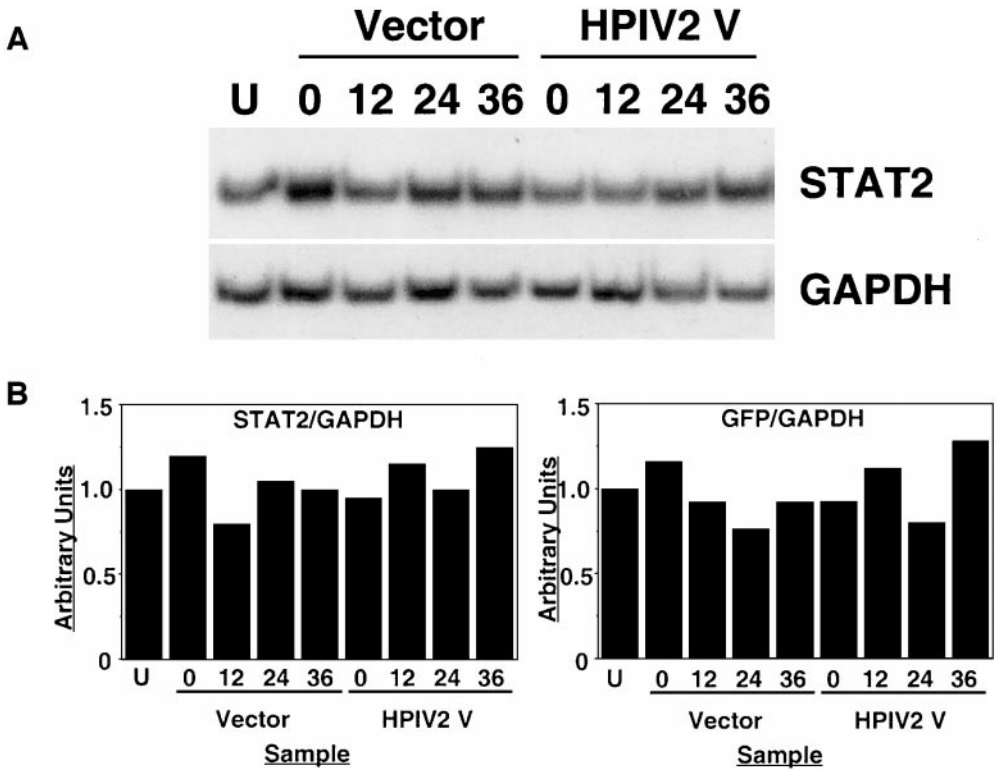

FIG. 4. Analysis of STAT2 mRNA levels upon V protein expression. A. RT-PCR was carried out using total RNA from cells transfected with empty vector or pRcCMV HPIV2 V as indicated. Each lane represents RNA isolated from an individual transfected 60-mm plate. U, untransfected cells; numbers indicate time (hpt) when RNA was isolated. B. Quantitation and normalization of (A) by phosphorimage analysis. The left graph shows the level of STAT2 normalized to the GAPDH control. The right graph shows normalized levels of cotransfected GFP mRNA.

mately 20–40% of control levels. Addition of CHX revealed a rapid loss of STAT2 to near completion by 48 hpt. Treatment of cells with IFN did not alter the V protein effect on STAT2 and neither STAT1 nor STAT3 levels were altered (not shown). This result indicates that steady-state STAT2 levels are the result of high stability of the bulk of STAT2 with approximately 20–30% of the total protein arising due to new protein synthesis. Expression of HPIV2 V protein results in destabilization of the pre-existing protein but has little effect on new STAT2 protein synthesis. These findings indicate that a primary action of HPIV2 V against STAT2 is posttranslational degradation.

Effect of proteasome inhibitors on HPIV2 V-mediated degradation

The results indicate that the effects of HPIV2 V protein on STAT2 were posttranslational. As SV5-mediated STAT1 degradation was blocked by proteasome inhibition in previously published experiments (Didcock *et al.,* 1999), it seemed probable that the mechanism of STAT2 degradation by HPIV2 might also involve the ubiquitin–proteasome pathway. To test this possibility, a proteasome inhibitor was used to treat transfected cells to determine the effect on HPIV2 V protein-mediated STAT2 degradation (Fig. 5C). When cells expressing HPIV2 V protein were treated with the proteasome inhibitor MG132, little difference was observed in the STAT2 steady-state level at 72 h posttransfection. However, concurrent inhibition of new protein synthesis with CHX treatment revealed that proteasome inhibition partially preserved STAT2 protein from the V-protein-mediated degradation. This result indicates only a minor role for proteasome-mediated degradation in the actions of the HPIV2 V protein against STAT2 and suggests that additional cellular mechanisms might also contribute to the V protein's activity.

DISCUSSION

The anti-viral state induced by IFN is a strong selective pressure for virus growth and replication. To evade the cellular IFN anti-viral effects, diverse virus types have created equally diverse strategies for bypassing this innate immune response. The paramyxoviruses SV5 and Sendai virus have been recently shown to cause a loss of cellular STAT protein accumulation, but by different mechanisms involving transcriptional inhibition catalyzed by Sendai virus C proteins (Garcin *et al.,* 1999, 2000) or posttranslational proteasomal degradation catalyzed by SV5 V protein (Didcock *et al.,* 1999). Our finding that infection of cells with HPIV2 leads to a loss of STAT2 steady-state protein accumulation is in general agree-

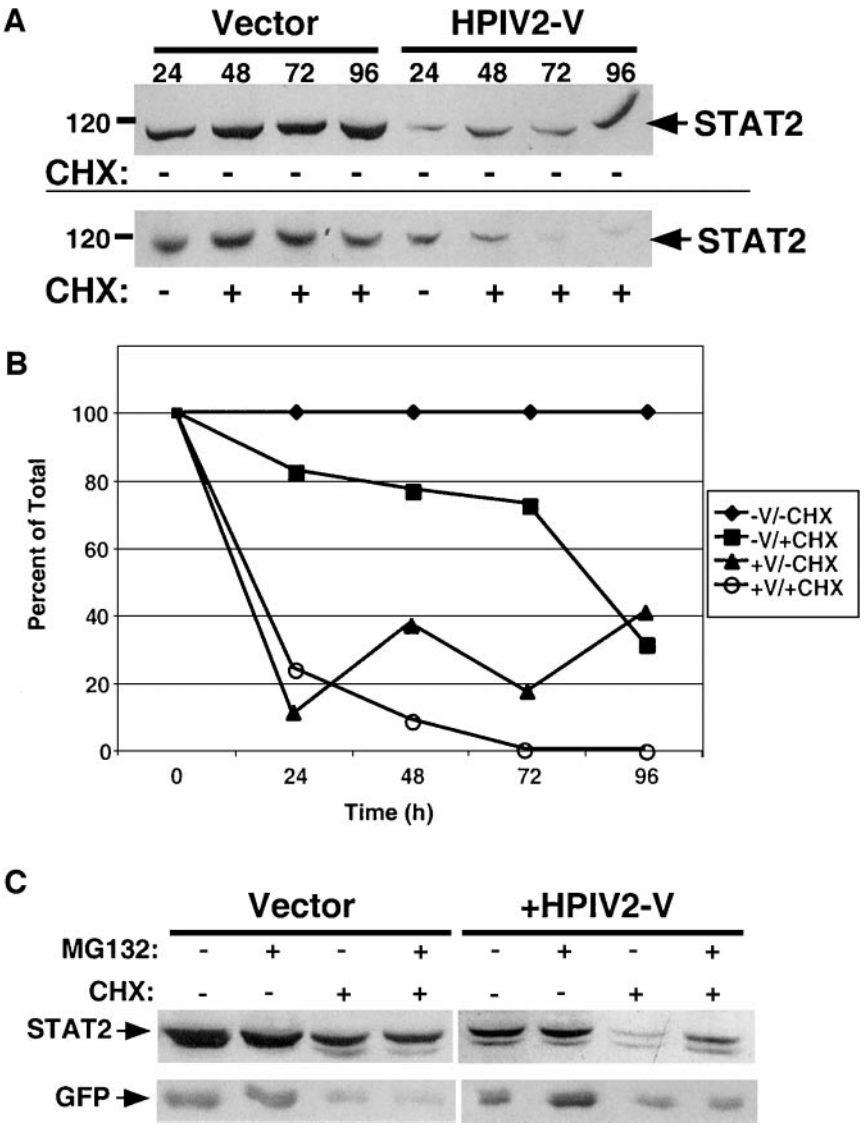

FIG. 5. Posttranslational degradation of STAT2 is induced by HPIV2-V protein expression. (A and B) Cells were transfected and treated with cycloheximide (CHX, 10 μg/ml) as indicated at 24 h posttransfection (hpt). A. Cell extracts were prepared at the indicated time points and analyzed for total STAT2 content by immunoblot. B. Immunoblotting results shown in (A) were quantitated by laser densitometry, normalized to the signal for STAT2 in vector-transfected, CHX-untreated control lanes, and plotted. Filled diamonds, vector control, CHX-untreated; filled squares, vector control, CHX-treated; filled triangles, HPIV2 V expression, CHX-untreated; open circles, HPIV2 V expression, CHX-treated. C. Effects of proteasome inhibitor on V-dependent STAT2 degradation. Cells were transfected with HPIV2 V expression plasmid and incubated at 24 h posttransfection with or without CHX. At 64 h posttransfection, 40 μM MG132 was added and extracts were prepared 8 h later. (Bottom) The effect of drug treatment on levels of protein from a control cotransfected GFP expression vector.

ment with a previous report (Young *et al.,* 2000). Here, we have analyzed the mechanism underlying HPIV2-mediated STAT2 inhibition by demonstrating that: (1) the HPIV2 V protein expressed in the absence of other viral proteins can target STAT2, (2) this loss of STAT2 induced by HPIV2 V protein expression results in defective IFN signaling to ISRE-containing promoter elements, (3) this loss of STAT2 is the result of V-protein-induced protein degradation, and (4) it is not the result of general transcriptional defects.

The finding of V-protein-mediated STAT2 protein degradation is directly analogous to SV5 degradation of STAT1 (Didcock *et al.,* 1999). We hypothesize that these two closely related viral proteins catalyze a similar reaction with different substrate specificity, possibly through interaction with and subversion of normal cellular proteolytic mechanisms. Proteins from other viruses are known that catalyze the proteolytic destruction of cellular targets by commandeering the cell's own degradation machinery. The two best characterized examples are from the study of human papilloma virus and human immunodeficiency virus and both appropriate the ubiquitin–proteasome system. The viral proteins physically interact with the E3 ubiquitin protein ligase enzymes by

forming a protein complex to bring novel targets to the proteasome. Degradation of the tumor suppressor p53 by the oncogenic HPV 16 and 18 E6 protein was the first example of such a mechanism (reviewed in Mantovani and Banks, 1999; Munger *et al.,* 1992). E6 functions by hijacking the cellular ubiquitin-dependent proteolytic machinery to induce p53 degradation. The viral E6 protein interacts directly with a cellular E3 enzyme, E6AP, causing novel recognition of p53 as a proteasome substrate. More recently, the HIV-1 Vpu integral membrane protein was demonstrated to catalyze the destruction of its own receptor, CD4, through interaction with a different E3 enzyme, βTrCP (Margottin *et al.,* 1998). While there is no amino acid sequence homology between the paramyxovirus V proteins and either the HPV-16 E6 or the HIV-1 Vpu, it is tempting to speculate that a similar mechanism has evolved to allow the paramyxovirus V proteins to mediate destruction of cellular STAT proteins that are otherwise long-lived and regulated by phosphorylation rather than transcriptional induction. Our results with chemical inhibition, however, suggest only a partial role for the proteasome per se in this reaction, leaving open the possibility for alternative mechanisms of V protein action. Future studies will be required to elucidate more precisely the mechanisms underlying HPIV2 V protein actions on STAT2 both in transfected cells and in the context of HPIV2 infections.

While the action of HPIV2 V protein appears to be similar to that of SV5 V, it differs from the IFN antagonizing effects of another paramyxovirus, Sendai virus. Expression of Sendai virus C proteins has been reported to cause a loss of STAT protein inducibility at the transcriptional level (Garcin *et al.,* 1999, 2000), but HPIV2 V protein apparently decreases the preexisting pool of STAT2 without altering the replacement of STAT2 by new protein synthesis. It is interesting to note that infection of cells with HPIV1 or HPIV3, which do not degrade STAT2, actually causes somewhat increased levels of STAT2 (see Fig. 1). It is possible that in addition to V protein actions against STAT2, HPIV2 infection also constraints host protein synthesis.

Many other viruses have evolved strategies to combat the IFN system, but other virus-induced mechanisms that cause proteolytic degradation of STATs are not known. Only one other viral protein was reported to similarly induce the loss of a STAT protein (Leonard and Sen, 1996). In this report, it was shown that chronic adenovirus E1A expression in Hela-derived cell lines corresponds with a decreased level of both STAT1 and IRF9/p48/ISGF3γ (two ISGF3 components). The mechanisms for either the STAT1 or the IRF9/p48/ISGF3γ loss remain uncharacterized, but the results with STAT1 were later found to differ between cell lines tested (Leonard and Sen, 1997). The loss of IRF9/p48/ISGF3γ, however, was more universal and E1A-expressing HT1080 cells transfected with IRF9/p48/ISGF3γ expression vectors were rescued from E1A's effects on IFN signaling. Other investigations suggest that E1A blocks IFN signaling by competing with STAT1 for limited basal transcription regulatory machinery, specifically the histone acetyl transferase, CBP/p300 (Bhattacharya *et al.,* 1996; Look *et al.,* 1998; Zhang *et al.,* 1996).

The effects of paramyxovirus-induced destruction of STAT proteins to evade IFN signaling are expected to influence not only innate cellular antiviral response, but also possibly the adaptive anti-viral immune responses. STAT1 is shared by both type I and type II (IFNγ) IFN signaling pathways (Horvath, 2000; Horvath and Darnell, 1997) and is also activated downstream of many other receptor and nonreceptor tyrosine kinases, serves as an accessory to several cytokine pathways, and functions in the modulation of cell death signals from apoptotic cytokine receptors (Kumar *et al.,* 1997; Wang *et al.,* 2000). Because of the more diverse activities of STAT1, the SV5 V protein is expected to have additional and possibly more severe cellular outcomes because its loss could influence additional cytokine signaling pathways in addition to type I IFN. By a similar argument, loss of STAT2 due to HPIV2 V protein expression is expected to result in a more specific loss of type I IFN signaling at the cellular level. Because murine and human STAT2 proteins are uncharacteristically divergent (Park *et al.,* 1999), the HPIV2 V protein might be responsible for limiting the host range of this virus to human cells (Ito *et al.,* 1989). The role of adaptive immune responses in HPIV2 infection represents a challenge for the future and will certainly provide insight into the biology of this human pathogen.

MATERIALS AND METHODS

Cell culture and virus infection

Human 293T, 2fTGH, and monkey CV1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL) and supplemented with 10% Cosmic calf serum (Hyclone). For protein synthesis inhibition experiments, specific cell lines in 60-mm dishes were treated with 10 μg/ml of cycloheximide (Sigma). For proteasome inhibition, Z-LLL-CHO (MG132; Sigma) was added to the medium to 40 μM final concentration for an 8-h exposure period (longer exposure times are cytotoxic). Treatment with human recombinant IFNα (1000 U/ml) was started at 12–16 h posttransfection for 6 h. Stocks of human parainfluenza viruses types 1, 2, and 3 were prepared and used as described previously (Ah-Tye *et al.,* 1999).

Plasmid DNAs, transient transfections, and reporter gene assays

Isolation of the HPIV2 V ORF was carried out by standard PCR methods (GDP, unpublished). Briefly, mRNA was harvested by oligo dT chromatography from CV-1

cells infected with the Greer strain of HPIV-2. The V ORF was amplified by RT-PCR using Superscript RT and Taq polymerase. The following primers were used:

HP2-P5′,
5′-GCGAATTCGTGATCAGATAACCTCCA-CACCAGAATCATAC-3′;
SACI-3′ vRNA sense,
5′-GCCGGTACCTTTAAGAGCTCAAT-GATCTCCTTCACATTCTCCGC-3′;

The PCR product was restricted with *Eco*RI and *Asp*718 and cloned into the same sites of pGEM3 (Promega). Three independent clones were sequenced and found to match the published Toshiba strain sequence except for a single nucleotide substitution (an A to G at base 239 on the published Toshiba sequence (Ohgimoto *et al.,* 1990)). This substitution converts a threonine to an alanine at position 56, possibly a strain-specific allelic difference. It may be relevant that the alanine at position 56 actually matches better to the SV5 V protein sequence.

The pGEM3 plasmid containing the Greer strain HPIV2 V ORF was the template for a second amplification of the V ORF insert by PCR using Vent Polymerase (NEB, Beverly, MA). The following primers were engineered to add flanking *Not*I and *Apa*I restriction endonuclease recognition sites to the V cDNA:

5′-GGGCGGCCGCGAATTCGTGATCAGATAAC-3′
5′-GGGGGCCCAAGAGCTCAATGATCTCC-3′.

The resulting PCR product was ligated into a *Not*I–*Apa*I restricted pRcCMV vector (Invitrogen) for mammalian expression and the resulting construct was verified by restriction mapping and nucleotide sequencing.

For transient cDNA transfection, cell monolayers were grown to 60–80% confluency in 60-mm dishes. Transfection of cells with cDNAs was carried out by standard $CaPO_4$ transfection procedures and assayed at various times after transfection (Ausubel *et al.,* 1994). Transfection of 293T cells by this method is highly efficient, resulting in typical transfection efficiencies of 95–100% as determined by FACS analysis of GFP-transfected cells (JFL and CMH, unpublished). Nonetheless, in all experiments, cotransfected controls were included to determine the uniformity and efficiency of the transfection and to verify that all plates in a given experiment were equally transfected. For cell lysate analysis, 1 μg of a control EGFP expression plasmid was included for evaluation of transfection efficiency by inverted fluorescence microscopy prior to lysis. For reporter gene assays, 1 μg of a control CMV-LacZ plasmid was added as a control for the transfection procedure and luciferase values were normalized to β-gal activity. In addition, 5 μg of a reporter gene containing five copies of the ISG54 ISRE element upstream of a TATA box and firefly luciferase ORF plasmid was included. After 16 h, transfection medium was replaced with fresh medium supplemented with 10% Cosmic calf serum. Cells were then harvested in an appropriate lysis buffer and analyzed either by SDS–polyacrylamide gel electrophoresis or by measuring luciferase activity according to the manufacturer's protocol (Promega).

Extracts, antibodies, and immunoblotting

Antiserum specific for STAT1 or STAT2 were obtained from Santa Cruz Biotechnology, antibodies specific for GFP were obtained from Clontech, and an antibody to HPIV2-infected cell extracts was obtained from Whitaker Biochemicals. Cell extracts were prepared as described (Shuai *et al.,* 1994). Cells were washed and scraped into ice-cold PBS and pelleted at 1500 rpm. Subsequent cell pellets were lysed in 2 vol of whole cell extract buffer (50 mM Tris, pH 8.0, 280 mM NaCl, 0.5% IGEPAL, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM DTT) supplemented with a protease inhibitor cocktail (Complete, Boehringer Mannheim) for 15 min. Cellular debris was pelleted at 14,000 rpm and the supernatant was analyzed directly or stored at −80°C. Samples were added to SDS–gel loading buffer, boiled for 5 min, and loaded directly onto SDS–polyacrylamide gels (7%, 12%). Gels were transferred to nitrocellulose membranes and detected with specific antibodies to the C-terminal portions of STAT1 and STAT2. Protein–antibody interactions were detected using secondary antiserum conjugated horseradish peroxidase and enhanced chemiluminescence reagents (NEN Life Sciences).

Analysis of mRNA

RT-PCR reactions were carried out as described previously (Horvath *et al.,* 1996). Total cellular RNA was isolated with Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. First-strand cDNAs were synthesized from DNAse-I-treated total RNA by use of SuperScript II reverse transcriptase (Gibco-BRL) and random antisense primers according to the manufacturer's instruction. Diluted cDNAs were subjected to PCR amplifications with [α-^{32}P]dCTP and primers specific for glyceraldehyde-3-phosphatase dehydrogenase (5′-GTGAAGGTCGGAGTCAAC-3′ and 5′-TGGAATTTGCCATGGGTG-3′) GFP 5′-ACGTAAACGGCCACAAGTTC-3′ and 5′-AAGTCGTGCTGCTTCATGTG-3′, or STAT2 (5′-GAAGATCTGAAACTCTCAATGAACTGG-3′ and 5′-GGGAATTCCCTAGGGAGCTCTGATGCAGG-3′).

PCR-amplified products were separated on a 5% polyacrylamide gel, and autoradiography was performed.

Samples were quantitated with a Storm phosphorimage analyzer and ImageQuant software (Molecular Dynamics).

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